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Effects of omega-3 fatty acids on complement-mediated glomerular epithelial cell injury

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Effects of omega-3 fatty acids on complement-mediated glomerular epithelial cell injury. To define the mechanisms by which fish oil protects rats with passive Heymann nephritis (PHN) from proteinuria *in vivo*, we investigated whether omega-3 fatty acid substitution of glomerular epithelial cells (GEC) in culture alters their susceptibility or response to complement-mediated sublethal injury. The results show that GECs can be cultured under conditions that effectively incorporate omega-3 or omega-6 fatty acids into membrane phospholipids without causing toxicity. Under these conditions, sublethal injury with anti-Fx1A and C5b-9 stimulated a 6.6-fold increase in TxA_2 production by GECs substituted with arachidonic acid (AA, omega-6) but no increase was detected in eicosapentaenoic acid (EPA, omega-3) substituted cells. Sublethal cell membrane injury was of equal severity in both groups as measured by the release of preloaded biscarboxyethyl carboxyfluorescein and by the transepithelial flux of albumin. In addition, omega-3 and omega-6 fatty acid substituted cells showed similar increases in diacylglycerol mass in response to sublethal injury by C5b-9, suggesting that omega-3 incorporation did not limit phospholipid (PL) hydrolysis by PLC. From this we can conclude that the protective effect of fish oil in PHN does not appear to result from the preservation of GEC integrity but is likely related to changes in the production of lipid mediators.

The passive Heymann nephritis (PHN) model of membranous nephropathy in rats is characterized by antibody-mediated and complement-dependent glomerular epithelial cell injury [1] accompanied by a substantial increase in glomerular eicosanoid production [2–4]. Several [4–8], though not all [2], studies using pharmacological blockers further indicated that the stimulated release of eicosanoids by injured glomeruli contributes to the magnitude of proteinuria in both active and passive Heymann nephritis. Related observations have been made in glomerular epithelial cells grown in culture [9]. Sublethal quantities of the complement membrane attack complex, C5b-9, activate phospholipases C and A_2, which initiates a cascade of cell signaling events [10, 11] and stimulates the production of eicosanoids and other inflammatory mediators [12–15]. Moreover, certain of these events, particularly calcium mobilization and activation of protein kinase C, appear to contribute to the ability of these [16] and other cells [17] to withstand an otherwise lethal attack by C5b-9.

Based on the foregoing observations we reasoned that substituting omega-3 fatty acids into cell membrane phospholipids by feeding rats a fish oil enriched diet might ameliorate injury in PHN by altering the profile of glomerular eicosanoids. Indeed, we found that a fish oil enriched diet abrogated the level of proteinuria in rats with PHN and markedly reduced the glomerular production of dienoic eicosanoids [3]. While these results suggested that the beneficial effects of fish oil in PHN are due to a reduction in TxA_2, and production instead of TxA_3, they did not discount the possibility of a direct effect of omega-3 fatty acids on the susceptibility or response of GECs to complement-mediated injury.

In the studies reported here we aimed to establish if omega-3 fatty acid substitution protects GECs from sublethal complement-mediated injury and, if not, determine whether it alters their response to such injury. Our results show that the major omega-3 fatty acid, eicosapentaenoic acid (EPA), can be effectively incorporated into GEC membrane phospholipids but it does not alter the susceptibility of the cells to C5b-9. On the other hand, substitution of EPA (omega-3) for arachidonic acid (omega-6) markedly reduces the output of dienoic eicosanoids, particularly TxA_2.

Methods

Rat glomerular epithelial cell culture

Passaged rat GECs from a continuous cell line [9] were used for all experiments. The epithelial nature of these cells was established by their polygonal shape and cobblestone appearance at confluence, cytotoxic susceptibility to low doses of aminonucleoside of puromycin, positive immunofluorescence staining for cytokeratin, and presence of junctional complexes on electron microscopy [9]. Additionally, these cells secrete collagen and laminin in a polarized fashion [18], express complement regulatory proteins [19], $\alpha 3\beta 1$ integrins [20, 21], and antigens that are present on rat GECs and that contribute to subepithelial immune deposits *in vivo* [22]. Therefore, these cells have several characteristics displayed by visceral GECs *in vivo*. Experiments were completed with cells between passages 19 and 30.

The cells were grown in standard K1 medium [13] or K1 supplemented with fatty acids. Standard K1 medium consists of 47% Dulbecco's modified Eagle's Medium (GIBCO, Grand Island, NY, USA), 47% Ham's F10 (GIBCO), 4.9% Nuserum (Collaborative Research, Bedford, MA, USA) and 0.1% hormone mix. The hormone mix contains hydrocortisone (18 ng/ml), insulin (5 μg/ml), prostaglandin E_1 (25 ng/ml), selenium (Na_2SeO_2, 1.7 ng/ml), transferrin (5 mg/ml) and 3,5,3′-triiodothyronine (T_3, 0.325 ng/ml) (Sigma Chemical Company, St Louis, MO, USA).

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Fatty acid supplementation of GECs in culture

To determine if GECs could thrive in fatty acid supplemented medium and to establish the maximum amount of fatty acid that could be tolerated, they were grown in K1 medium or K1 supplemented with 5 to 30 μM of linoleic acid (LA, 18:2, omega-6), arachidonic acid (AA, 20:4, omega-6), EPA (20:5, omega-3), oleic acid (OA, 18:1, omega-9), or palmitic acid (PA, 16:0) (NuChek Prep, Elysian, MN, USA) for 24, 48 or 72 hours. Viability of GECs plated with K1 medium containing 10% decomplemented calf serum (K1/CS) or K1/CS supplemented with 50 μM AA or EPA was also measured. The fatty acids were added to the medium in the form of the sodium salt. Stock fatty acid was added to 1 N NaOH in a glass test tube and quickly dried under nitrogen. Warm water was added to dissolve the fatty acid sodium salt and the solution was immediately transferred into the K1 medium. Fresh medium was prepared in this way for all experiments. GEC viability was determined by the uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in collagen coated 96-well culture dishes (Costar, Cambridge, MA, USA) as previously described [20, 23]. MTT, a tetrazolium salt, is taken up and converted to a visible reaction product in the mitochondria of live cells. Optical density was measured on an ELISA plate reader (model MR 5000; Dynatech, Chantilly, VA, USA) using a test wavelength of 570 nm and a reference wavelength of 630 nm. MTT uptake was determined from the OD 570/630 ratio, which is linearly related to the number of viable cells [20].

Incorporation of supplemented fatty acids into the four major classes of GEC membrane phospholipids: phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) was determined isotopically by thin layer chromatography (TLC) as previously described [10]. GECs were grown to confluence in 35 mm wells (6 wells per plate; Costar) in the presence of ^{3}H-EPA or ^{14}C-LA (New England Nuclear, Boston, MA, USA) at a concentration of 1 μCi/2.5 ml of medium. The radiolabeled fatty acids were added at the time of plating together with 15 μM LA or EPA supplemented K1 medium. After 24, 48 and 72 hours, the medium was replaced with 0.5 ml of ice-cold methanol and the cells were scraped into a glass tube containing the supernatant and acidified with formic acid (final concentration 0.2%, vol/vol). The lipids were extracted into 0.75 ml of chloroform-methanol (1:1.2) followed by 0.75 ml of chloroform. The solution was vortexed, centrifuged at 3,000 rpm for 10 minutes, the upper aqueous phase aspirated, and the remaining lower lipid phase evaporated under nitrogen. The organic phase was redissolved in a small amount of chloroform and spotted onto heat-inactivated LK5-DF silica gel TLC plates (Whatman, Clifton, NJ, USA) alongside appropriate phospholipid standards. The phospholipid classes were separated with a chloroform-methanol-acetic acid-water (50:37.5:3.5:2) solvent. The lipid bands were visualized with iodine vapor and those of interest were scraped separately into scintillation fluid and counted in a β-scintillation counter. The counts in the four phospholipid fractions were expressed as a percentage of the total radioactivity in each sample.

The uptake of individual fatty acids into GEC phospholipids was further analyzed by gas chromatography. GECs were grown in K1 medium alone, K1 medium containing 15 μM of LA, EPA or AA, or K1 medium supplemented with 10% decomplemented calf serum (as a source of AA) or 10% decomplemented calf serum enriched in EPA. To enrich with EPA, the calf serum was supplemented with 100 μM EPA in 0.1% fatty acid free bovine serum albumin and dialyzed for 48 hours against Hanks' balanced salt solution containing the same supplement. After growth for 48 hours in these media, cellular lipids were extracted as described above and resolved by TLC using a solvent system of hexane: ethyl-ether:glacial acetic acid (70:30:1). The phospholipid fraction was identified with co-chromatographed standards, scraped, and eluted from the gel with a 1:1 chloroform:methanol solvent. The fatty acids were transmethylated using BF_3/CH_3OH (Supelco, Bellafonte, PA, USA) [24]. The fatty acid methyl esters were separated on a 100 meter silica capillary column (RTX 2330; Restek, Bellefonte, PA, USA) packed with 90% biscyanopropyl and 10% phenylcyanopropyl using a 5890 Hewlett Packard separation system (Hewlett Packard Co., Avondale, PA, USA) equipped with a flame ionization detector (Dr. E. Siguel, Nutrition Unit, Boston University Medical Center, Boston, MA, USA). The fatty acid peaks were identified according to authentic standards (NuChek Prep) and quantification was performed using peak integration software (Justice Innovation, CA, USA). The results are expressed as a percentage of the total fatty acids in each sample.

Antibody and complement components

The complement fixing nephritogenic γ1 subclass of sheep anti-Fx1A IgG was used to sensitize the cells to complement. The non-complement fixing γ2 subclass of anti-Fx1A or nonimmune sheep IgG were used as controls. The γ1 and γ2 subclasses of sheep anti-Fx1A IgG were separated and purified by ion exchange chromatography as described [25]. Freshly donated normal human serum (NHS) was used as the source of complement. Previous studies have shown that sublethal injury induced in this way is entirely dependent upon assembly of C5b-9, the membrane attack complex of complement [9]. Heat-inactivated human serum (HIS, 56°C for 30 min) served as the negative control.

Measurement of complement-mediated sublethal cytotoxicity

Sublethal injury to GECs sensitized with anti-Fx1A and exposed to complement was assessed by the release of cell-incorporated biscarboxyethyl carboxyfluorescein (BCECF, molecular weight 820; Molecular Probes, Eugene, OR, USA) as previously described [9]. GECs were grown for 24 or 48 hours in 24 well plates (Costar) containing K1 medium alone or K1 medium supplemented with 15 μM fatty acid or 10% AA- or EPA-enriched calf serum as described above. Fatty acid supplements included AA, LA, EPA, PA, or OA. After 24 or 48 hours, the cells were washed with PBS and simultaneously loaded with 8 μM BCECF and sensitized with 2 mg/ml of γ1 anti-Fx1A for 40 minutes at 22°C on a microtiter plate shaker. Control cells were incubated with the same concentration of the non-complement fixing anti-Fx1A γ2 subclass or normal sheep IgG. After washing with PBS, experimental GECs were exposed to 5% NHS and control GECs to HIS for 40 minutes at 37°C. The supernatants were collected into individual microcentrifuge tubes. Aliquots of supernatant were also saved for thromboxane analysis (see below). The cells were washed with PBS and then lysed with mellitin (Sigma), 200 μg/ml in PBS, for 30 minutes at 37°C. The lysates were collected into individual tubes and, together with the supernatants, centrifuged for five minutes at 5,000 rpm. BCECF released into the

supernatants was quantified spectrofluorimetrically (Perkin-Elmer Co.) using an excitation wavelength of 506 nm and an emission wavelength of 530 nm. Specific BCECF release was calculated from the equation $(E - S)/(M - S) \times 100$, where E is experimental release, S is spontaneous release and M is maximum release (E plus melittin-induced release). Spontaneous release of BCECF was determined using supernatants from GECs incubated with PBS prior to lysis with mellitin.

Measurement of transepithelial albumin flux

Substituted and non-substituted cells were grown to confluence on 12 mm diameter collagen precoated polycarbonate filters (pore size 3.0 μm) suspended in multiwell plates (Costar). The optimal plating density and time taken for the cells to grow to confluence was defined in preliminary experiments. At confluence, rat albumin (50 μg/ml) was added to the lower chamber and basal albumin flux was measured by sampling from the upper chamber after 24 hours. γ1 anti-Fx1A and NHS or γ2 anti-Fx1A and HIS were then added to the lower chamber and samples were collected from the upper chamber at various time points over a further 24 hours period. The concentration of albumin in medium collected from both upper and lower chambers was measured by sandwich ELISA using Microtiter plates (Falcon, Becton-Dickinson, Oxnard, CA, USA) precoated with 100 μl of 20 μg/ml sheep anti-rat albumin (Cappel Research Products, Durham, NC, USA) [18]. Samples were diluted 1×10^6 and the reaction was developed with biotin-conjugated anti-rat albumin (4 μg/ml) (Cappel) and an avidin-peroxidase detection system (Sigma). Rat albumin (Sigma), diluted in the same buffer, was used as the standard.

Thromboxane production by GECs in response to complement-mediated injury

The concentration of TxB_2, the stable metabolite of TxA_2, in GEC-conditioned medium was measured by radioimmunoassay. The supernatants of GECs grown to confluence in K1 medium or in K1 supplemented with 15 μM AA or EPA and exposed to γ1 anti-Fx1A and NHS (experimental) or to the same concentrations of normal sheep IgG or γ_2 anti-Fx1A and HIS (control) for one hour were used for this assay (see above). In addition, to assess the ability of EPA to compete with AA, confluent non-substituted cells in multiwell plates were exposed to γ1 anti-Fx1A (2 mg/ml) and normal human serum for one hour at 37°C in the presence of a fixed concentration of AA (30 μM) and an increasing concentration of EPA (0 to 240 μM). Medium was then collected and assayed for TxB_2.

A 100 μl aliquot of GEC-conditioned medium or TxB_2 standard was incubated with 100 μl specific antiserum (Sigma) and 100 μl ^3H TxB_2 in buffer (1.21 g/liter Tris, 6.16 g/liter NaCl, pH 7.4) for one hour at 4°C. Unbound ligand was precipitated by addition of dextran-coated charcoal and the radioactivity in the supernatant was measured in a β liquid scintillation counter. The concentration of TxB_2 was calculated from a standard curve. The cross reactivity of other arachidonate metabolites with anti-TxB_2 at 50% displacement was as follows: < 0.1%: PGA_1, PGA_2, PGB_1, PGB_2, PGD_2, PGE_1, PGE_2, $PGF_{1\alpha}$, 6-keto-$PGF_{1\alpha}$, 13,14-dihydro-15-keto-PGE_2, 13,14-dihydro-15-keto-$PGF_{2\alpha}$; 0.5%: $PGF_{2\alpha}$. Cross reactivity with the trienoic thromboxane class (TxB_3) was not determined by the manufacturer and was therefore, unknown.

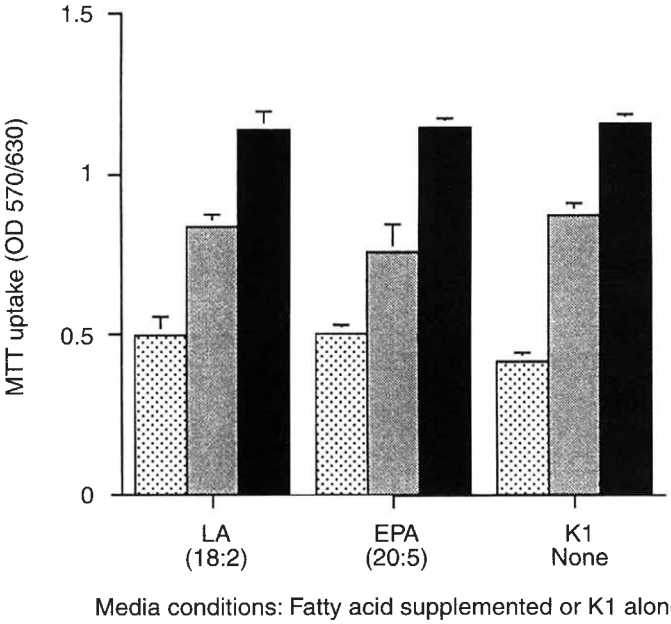

Fig. 1. *GEC growth and viability in the presence of fatty acid supplements.* Cells were grown in the presence of 15 μM linoleic (LA, omega-6) or eicosapentaenoic (EPA, omega-3) acid or standard, unsupplemented low serum medium (K1). The number of viable cells was measured in parallel plates after 24 (▨), 48 (▩) and 72 (■) hours by the uptake of MTT (**Methods**). Values are mean ± SEM (N = 6/condition at each time point).

Diacylglycerol production by GECs in response to complement-mediated injury

GECs were plated in 6 well plates (Costar) for 48 hours in K1 medium or K1 supplemented with the sodium salt of either 15 μM AA, EPA, LA, OA or PA. After 48 hours, experimental and control GECs were sensitized with 2 mg/ml anti-Fx1A for 40 minutes at 22°C as described above. This was followed by an additional 2 to 10 minutes incubation of experimental GECs with 5% NHS and control cells with 5% HIS. The final incubation was terminated with the addition of 750 μl ice-cold methanol. GEC lipids were then extracted as described earlier. Diacylglycerol (DAG) mass was determined in each sample with an assay that utilizes sn-1,2-DAG kinase extracted from *Escherichia coli* (Lipidex, Westfield, NJ, USA) according to the method developed by Preiss [26]. The assay employed ^{32}P-ATP as substrate and the mass of sn-1,2-DAG was determined by measuring the radioactive phosphatidic acid product, separated from the total lipid pool by TLC, and identified with a cochromatographed phosphatidic acid standard (Sigma). DAG mass was calculated from a curve of DAG standards measured in the same assay. The results are expressed as nanograms of DAG per well and as percent increase of DAG production according to the equation: (mean experimental)/(mean control) × 100.

Statistical analyses

Analyses were done with StatView™ 512+ (Brainpower, Inc., Calabasas, CA, USA). Analysis of variance (ANOVA) was used to test for differences between multiple groups, and differences between groups over time was tested by two-way ANOVA for repeated measures. A two-tailed t-test was used comparisons

Table 1. Incorporation of radiolabeled fatty acids into GEC phospholipids

	[^{3}H] or [^{14}C] fatty acid incorporated into phospholipids *percentage of total radioactivity*					
	EPA	LA	EPA	LA	EPA	LA
	24 hours[a]		48 hours[a]		72 hours[a]	
PI	7.0 ± 0.00	9.5 ± 0.75	6.3 ± 0.65	7.0 ± 0.50	12.0 ± 0.00	6.3 ± 0.65
PS	3.0 ± 0.00	6.8 ± 0.54	3.5 ± 0.25	4.0 ± 0.50	3.0 ± 0.00	6.0 ± 0.00
PC	8.5 ± 0.35	25.0 ± 0.65	7.5 ± 0.25	22.0 ± 0.89	10.5 ± 0.35	20.0 ± 0.21
PE	70.0 ± 0.00	40.0 ± 2.90	73.5 ± 0.43	49.5 ± 2.27	67.0 ± 0.71	49.0 ± 5.00

Abbreviations are: EPA, [^{3}H]-eicosapentaenoic acid; LA, [^{14}C]-linoleic acid; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

[a] Time in culture in the presence of EPA or LA

between two groups. A P value of < 0.05 was considered significant.

Results

Fatty acid substitution of rat GECs

As illustrated in Figure 1 for LA and EPA, the viability and proliferative ability of GECs were unaffected by adding omega-6 or omega-3 polyunsaturated fatty acids (PUFAs) to the growth medium for periods up to 72 hours at concentrations up to 15 μM. The viability and growth of cells grown in fatty acid concentrations of 18 μM and 30 μM for up to 48 hours were no different than in cells grown in standard K1 medium; thereafter, however, both the omega-6 and omega-3 PUFAs moderately inhibited the increase in MTT-positive cells indicating some cell attrition and/or a reduced rate of proliferation (data not shown). On phase contrast microscopy, cells exposed to concentrations of fatty acid greater than 15 μM were more vacuolated than K1 controls or cells exposed to the lower concentrations. Cell survival was not further improved by adding the antioxidant α-tocopherol (25 μM) or delipidated bovine serum albumin (1%) to the medium (results not shown). Therefore, in subsequent long-term studies (>24 hr), fatty acid concentrations were limited to 15 μM and the medium was not further modified. These conditions were also found to be appropriate for various other poly- and monounsaturated fatty acids including arachidonic, palmitic and oleic acids.

Addition of tracer amounts of tritiated fatty acids and analysis of membrane phospholipids by thin layer chromatography indicated that isotopic equilibrium was achieved within 24 hours. The distribution of EPA and LA incorporated into the major classes of phospholipids is shown in Table 1. From these analyses, the labeled omega-6 fatty acids derived from LA were preferentially incorporated into phosphatidylcholine and phosphatidylethanolamine whereas the labeled omega-3 fatty acids from EPA were mainly located in phosphatidylethanolamine Table 1. Uptake into phosphatidylinositol was a small but significant fraction of the total incorporation and was similar for the two fatty acids.

Gas chromatography of phospholipids from GECs grown in the presence of 15 μM AA or EPA showed significant incorporation of the respective fatty acids as compared to cells grown in standard K1 medium (Fig. 2). Although LA-substituted cells had significantly more LA than cells grown in K1 medium alone they did not show an increase in AA, which suggests that elongation of LA to AA (the major omega-6 fatty acid in the sn-2 position of phospholipids) is relatively inefficient in these cells. Therefore, several subsequent studies were conducted with AA instead of LA. The gas chromatographic analysis further revealed a baseline fatty acid profile that suggests essential fatty acid deficiency in cells grown in standard K1 medium containing approximately 2.5% fetal bovine serum (triene:tetraene ratio 2.35 ± 0.019) and that this profile is not substantially improved by the addition of 15 μM AA (triene:tetraene ratio 0.506 ± 0.018), LA (triene:tetraene ratio 1.515 ± 0.137) or EPA (2.03 ± 0.151). Therefore, to confirm that our results were not attributable to essential fatty acid deficiency, further studies were conducted in which the deficiency was corrected by growing the cells in K1 medium containing 10% calf serum enriched in AA or EPA. This increased the amount of AA and EPA in GEC phospholipids and improved the triene:tetraene ratio to <0.2. The gas chromatographic analysis of these cells is also shown in Figure 2.

Effects of fatty acid substitution on GEC susceptibility to complement-mediated injury

Figure 3 illustrates one of several experiments conducted to determine if the fatty acid composition of membrane phospholipids influenced the susceptibility of GECs to sublethal complement-mediated injury as measured by release of the fluoroprobe, BCECF. Exposure to complement-fixing γ1 anti-Fx1A and fresh human serum as the source of complement produced substantial release of BCECF as compared to control cells exposed to γ2 anti-Fx1A and heat-inactivated serum; however, the level of release was not significantly different in cells substituted with various fatty acids than in unsubstituted cells. In particular, EPA substitution did not render the cells more resistant to injury. AA substitution induced slightly more basal leakage of BCECF in control cells but this did not make them more sensitive to complement-mediated injury. Supernatants from this experiment were subsequently analyzed for thromboxane B_2 release (see below). Similar results were obtained in other experiments in which EPA-substituted cells were compared to cells substituted with LA, palmitic acid and oleic acid (data not shown).

To ensure that a significant effect of omega-3 fatty acid substitution was not masked by an underlying deficiency in essential fatty acids, similar experiments were conducted with GECs in which the deficiency had been corrected. As before, the results showed no difference between AA- and EPA-substituted and unsubstituted cells (Fig. 4). Similarly, in GECs exposed to a lethal dose of complement (10% fresh human serum), cell viability as measured by MTT uptake was not significantly improved by EPA substitution (data not shown).

The functional effects of complement-mediated GEC injury were further quantified by measuring transepithelial albumin flux.

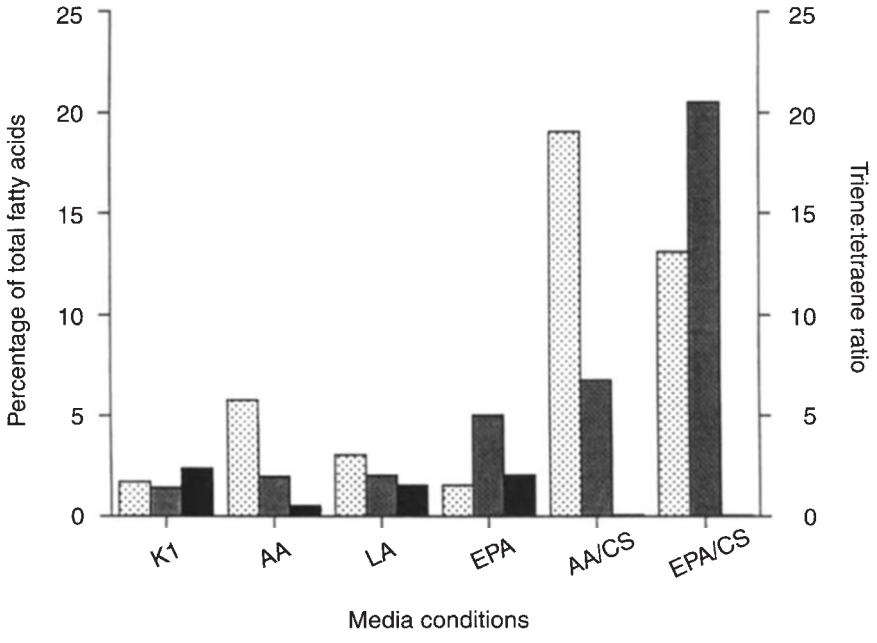

Fig. 2. *Gas chromatographic analysis of GEC membrane phospholipids after fatty acid supplementation.* Cells were grown for 48 hours in K1 medium alone, K1 medium containing 15 μM of arachidonic (AA, omega-6), linoleic (LA, omega-6) or eicosapentaenoic (EPA, omega-3) acid, or K1 medium supplemented with 10% decomplemented calf serum (AA/CS) or 10% decomplemented calf serum enriched with EPA (EPA/CS). The lightly hatched bars (▨) represent omega-6 and the darkly hatched (▩) bars represent omega-3 fatty acids. The black bars (■) are the ratio of fatty acids (20:3:20:4) and are barely visible in the AA/CS and EPA/CS lanes (values > 0.2 are indicative of essential fatty acid deficiency). Values are the average of 2 to 3 determinations. The axis on the left represents the sum of all omega-6 or omega-3 fatty acids expressed as a percentage of total fatty acids. The axis on the right indicates the triene:tetraene ratio.

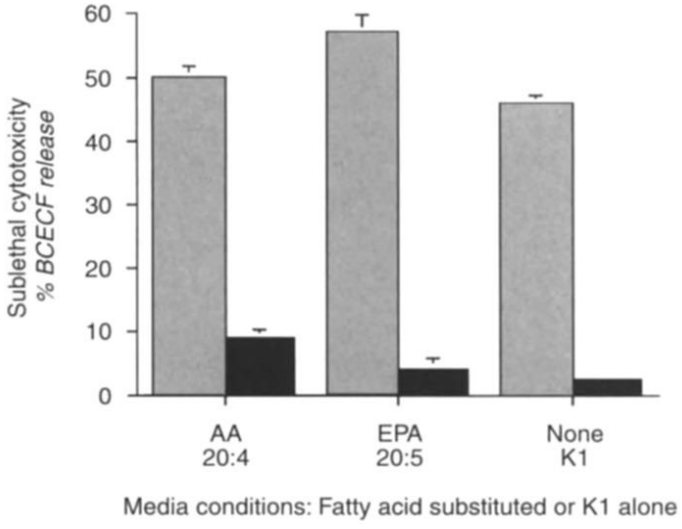

Fig. 3. *Effect of fatty acid substitution on sublethal complement-mediated cytotoxicity.* GECs were grown for 48 hours in K1 medium alone or K1 medium containing 15 μM of AA or EPA. They were then loaded with 8 μM BCECF and sensitized with 2 mg/ml of γ1 anti-Fx1A (experimental) or non-complement fixing γ2 anti-Fx1A for 40 minutes at 22°C. Experimental GECs (▨) were exposed to 5% fresh serum and control GECs (■) to heat-inactivated serum for 40 minutes at 37°C. Specific BCECF release, expressed as a percentage of total releasable BCECF, is significantly different between experimentals and controls within each group ($P < 0.001$) but there is no significant difference between groups (ANOVA). Values are mean ± SEM ($N = 6$/group).

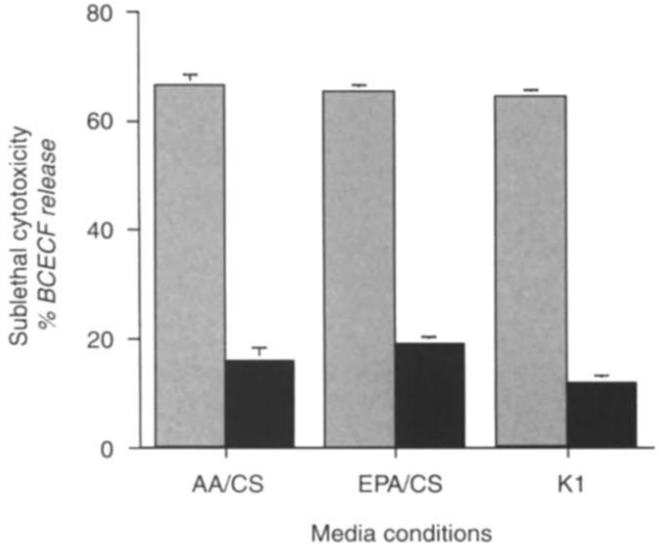

Fig. 4. *Effect of restoring GEC fatty acids with AA or EPA on sublethal complement-mediated cytotoxicity.* GECs were grown for 48 hours in K1 medium containing 10% decomplemented calf serum alone (K1/CS) or enriched in AA (AA/CS) or EPA (EPA/CS). At the time of plating, 50 μM AA or EPA was added to AA/CS or EPA/CS, respectively. The cells were then loaded with BCECF and exposed to antibody and complement and studied as described in Figure 3. BCECF release is significantly different between experimentals (▨) and controls (■) within each group ($P < 0.001$) and is slightly but significantly lower in K1/CS controls ($P < 0.01$) but there is no significant difference between experimental groups (ANOVA). Values are mean ± SEM ($N = 3$/group).

GECs grown to confluence on collagen-coated permeable supports provide an effective barrier to the passage of macromolecules. This barrier was rapidly destroyed by sublethal injury by complement (Fig. 5), however, the rate of albumin leakage was not different in omega-3 or omega-6 substituted cells than in unsubstituted cells.

Effects of fatty acid substitution on the response of GECs to sublethal complement-mediated injury

Increased release of eicosanoids is an established sublethal cytotoxic effect of complement. This was verified in these studies by a higher level of thromboxane B_2 production by GECs

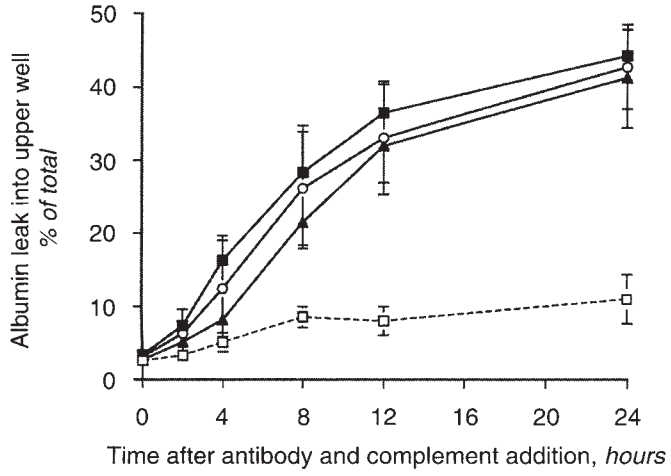

Fig. 5. *Effect of fatty acid substitution on transepithelial flux of albumin.* GECs were grown to confluence on collagen coated polycarbonate filters suspended in multiwell plates in the presence of K1 medium alone or K1 medium containing 15 μM of LA or EPA. At confluence, rat albumin (50 μg/ml) was added to the lower chamber and basal albumin flux was measured by sampling from the upper chamber after 24 hours. γ1 anti-Fx1A and fresh serum (experimental) or γ2 anti-Fx1A and heat-inactivated serum (control) were then added to the lower chamber and samples were collected from the upper chamber at various time points over a further 24 hour period. No significant increase in the flux of albumin was observed in control cells. Increased flux occurred in all experimental groups ($P < 0.001$) but was not significantly different between the groups (ANOVA). Values are mean of four separate experiments each using three replicate wells (SEM < 8%). Symbols are: (□) K1 control; (■) K1 experimental; (○) LA experimental; (▲) EPA experimental.

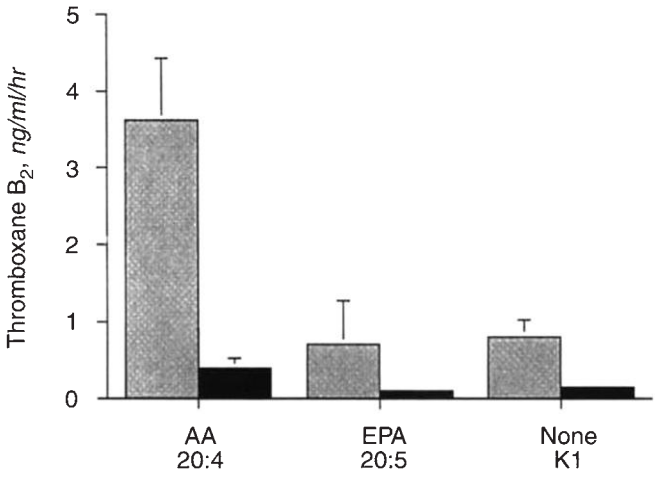

Fig. 6. *Effect of fatty acid substitution on complement-mediated thromboxane B_2 production.* The experimental conditions are described in the legend to Figure 3 except that TxB_2 was measured in the supernatants after one hour of incubation. TxB_2 production was increased in experimental cells (▨) under all media conditions as compared to controls (■; $P < 0.05$), but was significantly greater in AA-substituted than in K1 or EPA-substituted experimental cells ($P < 0.005$, ANOVA). Values are mean ± SD (N = 4/group) from a representative experiment.

sensitized with γ1 anti-Fx1A and exposed to complement than cells exposed to normal sheep serum and heat inactivated complement (Fig. 6). This difference was seen in all medium conditions, presumably reflecting the residual AA in membrane phospholipids, but was most evident in cells enriched with AA (Fig. 6). AA-enriched cells demonstrated a 6.6-fold greater level of thromboxane B_2 production than cells in standard K1 medium after stimulation with complement. On the other hand, despite the equivalent level of injury in EPA- and AA-substituted cells exposed to antibody and complement, there was substantially less thromboxane B_2 produced by EPA-substituted cells (Fig. 6).

Furthermore, EPA was able to competitively inhibit thromboxane B_2 production by complement stimulated GECs even in the presence of AA. In this experiment, medium recovered from non-substituted cells exposed for one hour to γ1 anti-Fx1A and complement in the presence of 30 mM AA contained 3.75 ± 0.32 ng/ml thromboxane B_2. Addition of EPA at the time of stimulation led to a concentration dependent decrease (65% at 120 mM) in thromboxane B_2 production by injured cells but did not completely block the production of this eicosanoid (Fig. 7). These results suggest that EPA competes effectively with AA as a substrate for cyclooxygenase.

Using trace labeling techniques, we have previously shown that there is increased diacylglycerol (DAG) production when GEC membrane phospholipids are hydrolyzed in response to sublethal injury by complement [10]. In these studies we measured the total amount of DAG released in response to complement because one cannot be certain that trace-labeled fatty acids of different classes are equally incorporated into the sn-2 position of membrane phospholipids. We confirmed, in several experiments, that anti-Fx1A and complement stimulate an increase in DAG mass but we also found an effect of serum alone when incubation was extended for 10 minutes or more (data no shown). Therefore, in the experiment shown in Figure 8, DAG production by GECs sensitized with anti-Fx1A was measured after two minutes of exposure to complement or heat-inactivated serum. DAG levels were higher in the experimental GECs than in the respective controls regardless of whether the cells were grown in standard K1 medium or K1 supplemented with 15 μM EPA or LA. The percent increase of DAG production in injured GECs over control GECs grown in K1 was 161%. The increases observed with omega-6 and omega-3 supplemented cells were 209% (LA) and 183% (EPA), respectively. Similar increases were seen with oleic (256%) and palmitic (204%) acid substituted cells (data not shown), however the mass of DAG released after complement-mediated injury did not differ between cells substituted with any of the various fatty acids.

Discussion

These experiments were specifically designed to determine if omega-3 fatty acid substitution of GECs in culture alters their susceptibility or response to complement-mediated sublethal injury. Our results show that omega-3 or omega-6 fatty acids can be effectively incorporated into membrane phospholipids of cultured GECs without causing toxicity. Under these conditions, sublethal injury with anti-Fx1A and C5b-9 stimulated a 6.6-fold increase in TxA_2 production by GECs substituted with AA (omega-6). On the other hand, no increase was detected in EPA (omega-3) substituted cells despite a similar degree of cell membrane injury as measured by the release of preloaded BCECF (a marker of sublethal cytotoxicity) and by the transepithelial flux of albumin.

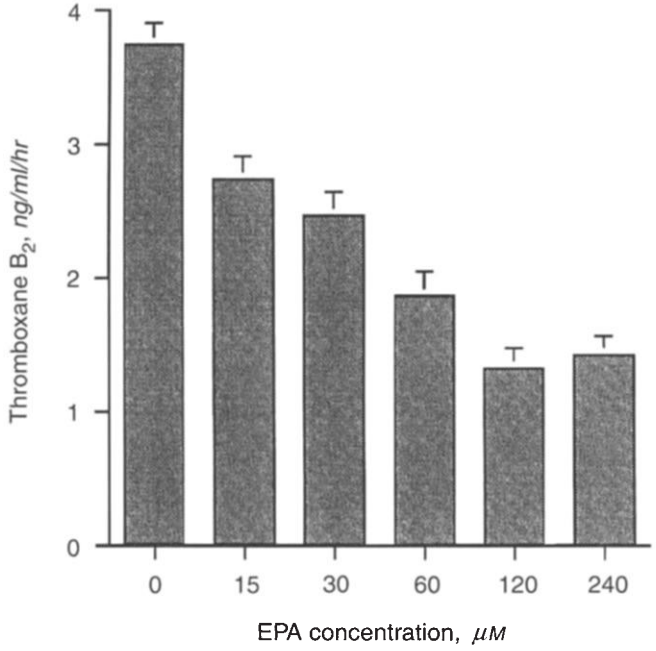

Fig. 7. *Dose-response effect of EPA on TxB_2 production by complement-stimulated AA-supplemented GECs.* Confluent non-substituted cells were exposed to γ1 anti-Fx1A (2 mg/ml) and normal human serum for one hour at 37°C in the presence of a fixed concentration of AA (30 μM) and an increasing concentration of EPA (0 to 240 μM). The medium was then assayed for TxB_2. A significant dose-related inhibition of TxB_2 production was observed at EPA concentrations up to 120 μM ($P < 0.05$, ANOVA). Values are mean ± SD ($N = 4$/dose).

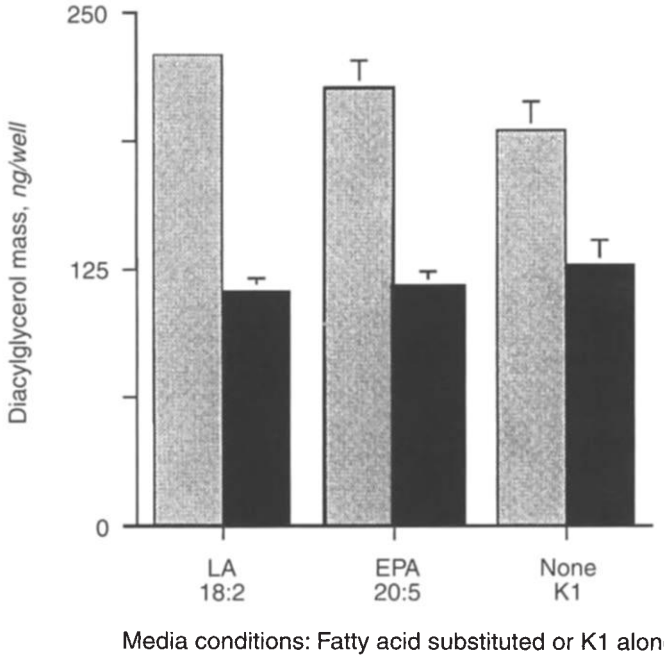

Fig. 8. *Effect of fatty acid substitution on complement-mediated diacylglycerol (DAG) production.* GECs were grown for 48 hours in K1 medium alone or K1 medium containing 15 μM of LA or EPA. They were then sensitized with 2 mg/ml of γ1 anti-Fx1A (experimental) or non-complement fixing γ2 anti-Fx1A (control) for 40 minutes at 22°C. Experimental GECs were exposed to 5% fresh serum and control GECs to heat-inactivated serum for two minutes at 37°C. DAG mass was then measured in lipid extracts of the methanol-lysed cells by the DAG kinase assay (**Methods**). DAG mass was significantly greater in experimental cells than in controls ($P < 0.001$) but did not differ between experimental groups (ANOVA). Values are mean ± SEM ($N = 3$ to 4/group).

In addition, omega-3 and omega-6 fatty acid substituted cells showed similar increases in diacylglycerol mass in response to sublethal injury by C5b-9, indicating that omega-3 incorporation did not limit phospholipid hydrolysis by PLC. From these findings we conclude that the protective effect of fish oil in PHN does not appear to result from the preservation of GEC integrity, but may be related to the changes in lipid mediators such as TxA_2, a potent vasoconstrictor eicosanoid that has been implicated in the pathogenesis of proteinuria [27].

In attempting to understand the mechanisms by which fish oil ameliorates proteinuria in experimental membranous nephropathy [3, 28], we were faced with two possibilities. The first would implicate a hemodynamic, pro-inflammatory or cytopathic effect of lipid mediators. It is well established that glomeruli isolated from such rats produce high levels of eicosanoids [2–4, 28]. It is particularly relevant to our present studies that high levels of TxA_2 are produced in response to complement-mediated injury [2] and are suppressed by fish oil [3]. We also found that selective blockade of thromboxane synthase suppressed the magnitude of proteinuria in an isolated perfused kidney model of PHN [5]. PLA_2-induced hydrolysis of EPA-substituted membrane phospholipids leads to production of trienoic prostanoids (for example PGI_3, PGE_3 and TxA_3) and pentaenoic leukotrienes (for example LTB_5) instead of dienoic prostanoids (PGI_2, PGE_2 and TxA_2) and tetraenoic leukotrienes (LTB_4). TxA_3 and LTB_5 are weak agonists. Consequently, TxA_3 is much less effective in stimulating platelet aggregation and vasoconstriction than TxA_2, and LTB_5 is a less potent leukocyte chemotaxin and stimulant of leukocyte-endothelial adhesion than LTB_4 [28]. TxA_3 is also a competitive inhibitor of TxA_2 [29], and LTA_5 competes for LTA_4-hydrolase [30], the rate-limiting enzyme for the conversion of LTA_4 to LTB_4, LTC_4 and LTD_4 [31]. Therefore, the benefits of fish oil might have been due to changes in the production of lipid mediators in response to complement-mediated cell injury.

The second possibility, and the one tested in these studies, predicts that omega-3 fatty acids afford direct cytoprotection against the attack by C5b-9. This concept is given credence by our studies of complement-mediated sublethal GEC injury *in vitro*, which disclosed an intracellular signaling cascade that involves phospholipid hydrolysis and results in the production of IP_3, DAG and eicosanoids, as well as the release and influx of calcium [10, 11]. In addition, *in vitro* studies have demonstrated that incorporation of omega-3 fatty acids into vascular smooth muscle inositol phospholipids [32], reduces agonist-induced phosphoinositide turnover, IP_3 generation and calcium transients [33, 34], suggesting that similar mechanisms may be at work in injured glomeruli. Therefore, by analogy to these findings, we reasoned that similarly treated GECs might exhibit reduced phospholipid hydrolysis and less membrane damage in response to complement. It is important to note, however, that some of the events activated by complement in GECs appear to contribute to their ability to withstand an attack by complement [16], perhaps by stimulating membrane vesiculation and shedding of C5b-9 complexes [35–38]. Therefore, we were aware that omega-3 fatty acids might aggravate injury by inhibiting these protective mechanisms or by

increasing GEC susceptibility to potential membrane damaging effects of oxidants or high levels of calcium [14, 39].

In support of a direct cytoprotective action, there is evidence that certain effects of fish oils cannot be readily attributed to their ability to modify lipid mediators. For example, fish oils or purified omega-3 fatty acids inhibit the proliferation of precancerous cells [40] and the oncogenic transformation of cultured fibroblasts [41]. They reduce cytokine and growth factor production by mononuclear [42] and endothelial [43] cells, and suppress the expression of leukocyte adhesion molecules on activated endothelial cells [44]. They also correct the hyperlipidemia of nephrotic rats [45] and abolish calcium-dependent ventricular fibrillation in cultured cardiac myocytes [46].

Our results are informative in several respects. In contrast to the observations in vascular smooth muscle cells [32–34], we found that altering the composition of membrane phospholipids had no appreciable effect on phospholipid hydrolysis (as measured by DAG release) in response to complement. In particular, omega-3 fatty acid substituted GECs released as much DAG as cells substituted with omega-6, monounsaturated and saturated fatty acids after stimulation with C5b-9. Not surprisingly, the stimulated release from unsupplemented cells was slightly lower than the other groups. Furthermore, sensitive functional assays of cell membrane and epithelial integrity did not demonstrate protection by omega-3 fatty acids. On the other hand, there was no evidence that polyunsaturated fatty acid supplementation aggravated injury. These findings are consistent with our *in vivo* observations, in which podocyte effacement and condensation of the actin cytoskeleton were seen to be similar in fish oil and safflower oil fed rats with PHN despite a reduction in proteinuria with fish oil [3].

Presubstitution with EPA markedly inhibited complement-stimulated TxB_2 release by GECs, consistent with the documented activation of PLA_2 by C5b-9 [11] and the ability of EPA released from the sn-2 position of membrane phospholipids to competitively inhibit the interaction of AA and cyclooxygenase. Moreover, addition of EPA at the time of injury suppressed TxB_2 release in a dose-dependent fashion. This is likely due, at least in part, to a direct action of free EPA that is rapidly translocated across the lipid bilayer into the cells where it competes with AA being released from membrane phospholipids (especially phosphatidylcholine) by complement-activated PLA_2 [15]. Therefore, the profound reduction in glomerular TxB_2 production observed in fish oil fed PHN rats [3] is likely due to the combined effects of phospholipid-associated and free omega-3 fatty acids. Although there may have been a concomitant rise in TxA_3, this cannot be ascertained without a specific assay.

We should also point out that these and our previous *in vivo* studies do not exclude the possibility that the benefits of fish oil in PHN are due, in part, to an effect on other lipid or non-lipid mediators. For example, it has been shown that glomerular LTB_4 production is significantly increased soon after anti-Fx1A injection and at the onset of complement-dependent proteinuria in PHN [4]. Further studies with pharmacological inhibitors demonstrated that LTB_4 contributes to the magnitude of proteinuria, and LTD_4 to the glomerular hemodynamic changes that characterize the autologous phase of PHN, and that the primary origin of these products of the 5-lipoxygenase pathway appears to be a small but significant infiltrate of mononuclear cells [8]. However, our own studies have consistently failed to reveal a role for leukocytes in the pathogenesis of GEC injury and proteinuria in the heterologous phase of PHN [1, 47], and there is no evidence as yet that GECs are capable of synthesizing leukotrienes [31]. Therefore, in studying the primary events responsible for this model of injury, namely complement-mediated GEC damage, we have focused on the eicosanoids produced by GECs *in vivo* and *in vitro*.

In conclusion, these studies indicate that podocytes injured by complement are the likely source of thromboxane released by glomeruli isolated from rats with PHN and that fish oil ameliorates proteinuria by switching eicosanoids to the omega-3 series, rather than by protecting their structural integrity.

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